

Targeted Recombinant Fusion Proteins of IFN γ and Mimetic IFN γ with PDGF β R Bicyclic Peptide Inhibits Liver Fibrogenesis *In Vivo*

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Abstract

Hepatic stellate cells (HSCs), following transdifferentiation to myofibroblasts plays a key role in liver fibrosis. Therefore, attempts to attenuate this myofibroblastic phenotype would be a promising therapeutic approach. Interferon gamma (IFN γ) is a potent anti-fibrotic cytokine, but its pleiotropic receptor expression leading to severe adverse effects has limited its clinical application. Since, activated HSC express high-level of platelet derived growth factor beta receptor (PDGF β R), we investigated the potential of PDGF β R-specific targeting of IFN γ and its signaling peptide that lacks IFN γ R binding site (mimetic IFN γ or mimIFN γ) in liver fibrosis. We prepared DNA constructs expressing IFN γ , mimIFN γ or BiPPB (PDGF β R-specific bicyclic peptide)-IFN γ , BiPPB-mimIFN γ fusion proteins. Both chimeric proteins alongwith IFN γ and mimIFN γ were produced in *E.coli*. The expressed proteins were purified and analyzed for PDGF β R-specific binding and *in vitro* effects. Subsequently, these recombinant proteins were investigated for the liver uptake (pSTAT1 α signaling pathway), for anti-fibrotic effects and adverse effects (platelet counts) in CCl₄-induced liver fibrogenesis in mice. The purified HSC-targeted IFN γ and mimIFN γ fusion proteins showed PDGF β R-specific binding and significantly reduced TGF β -induced collagen-I expression in human HSC (LX2 cells), while mouse IFN γ and mimIFN γ did not show any effect. Conversely, mouse IFN γ and BiPPB-IFN γ induced activation and dose-dependent nitric oxide release in mouse macrophages (express IFN γ R while lack PDGF β R), which was not observed with mimIFN γ and BiPPB-mimIFN γ , due to the lack of IFN γ R binding sites. *In vivo*, targeted BiPPB-IFN γ and BiPPB-mimIFN γ significantly activated intrahepatic IFN γ -signaling pathway compared to IFN γ and mimIFN γ suggesting increased liver accumulation. Furthermore, the targeted fusion proteins ameliorated liver fibrogenesis in mice by significantly reducing collagen and α -SMA expression and potentiating collagen degradation. IFN γ also induced reduction in fibrogenesis but showed significant decrease in platelet counts, which was restored with targeted proteins. These results suggest that these rationally designed proteins can be further developed as novel anti-fibrotic therapeutics.

Citation: Bansal R, Prakash J, De Ruiter M, Poelstra K (2014) Targeted Recombinant Fusion Proteins of IFN γ and Mimetic IFN γ with PDGF β R Bicyclic Peptide Inhibits Liver Fibrogenesis *In Vivo*. PLoS ONE 9(2): e89878. doi:10.1371/journal.pone.0089878

Editor: Rafael Aldabe, Centro de Investigación en Medicina Aplicada (CIMA), Spain

Received: October 1, 2013; **Accepted:** January 24, 2014; **Published:** February 24, 2014

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Funding: Work was supported by VICI grant-in-aid from Netherlands Organization for Scientific Research (NWO; <http://www.nwo.nl/>) and the Technical foundation (STW; <http://www.stw.nl/>). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: KP is scientific advisor and shareholder of BiOrion Technologies B.V., a company dedicated to the commercialization of drug carriers, including the carrier presented here. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

IFN γ is a pleiotropic homodimeric Th1 cytokine mainly produced by activated inflammatory cells and has been documented to be highly effective in viral, immunological and malignant diseases [1,2]. IFN γ (Interferon gamma-1b) has also been explored in clinical trials in patients suffering from liver fibrosis, renal fibrosis or idiopathic pulmonary fibrosis [3–6]. However, despite its promising effects *in vivo*, all clinical trials failed due to a lack of efficacy and unfavorable adverse effects [4,7,8]. The clinical application of this potent cytokine is nowadays limited to only a very few diseases. Many attempts have been made to prolong the half-life of IFN γ by PEGylation or by increasing its activity through slow release by incorporation in nanoparticles, elastomers, microspheres or liposomes [9–11]. These approaches have shown to be beneficial, but adverse effects due to the longer exposure of IFN γ to non-target tissues can still be detrimental. Therefore, targeted approaches leading to an increased therapeutic

efficacy without eliciting adverse effects would be ideal to treat slowly progressing chronic diseases [12].

Liver fibrosis, induced by viral infections (e.g hepatitis B and C), alcohol abuse, metabolic syndrome or genetic disorders, is characterized by an excessive accumulation of matrix proteins in the liver [13,14]. Worldwide millions of people, suffering from one of these disorders, are at risk of developing liver fibrosis. Currently, there are no effective and clinically approved anti-fibrotic therapy available and the treatment is mainly based on the removal of the underlying cause of the disease [15,16]. Liver transplantation is the only option for the patients suffering from advanced liver fibrosis or end stage liver cirrhosis.

Hepatic stellate cells (HSC) are the key pathogenic cells involved in the progression of liver fibrosis. These cells get activated following release of growth factors from damaged hepatocytes, kupffer cells and infiltrating inflammatory cells. These activated HSC are then transformed into proliferative and contractile myofibroblast-like cells that produce large amounts of extracellular

matrix (ECM) proteins leading to impairment of the structure and function of liver [17,18]. Among the potent anti-fibrotic therapeutic cytokines, Interferon gamma (IFN γ) is shown to be highly efficacious *in vitro* and *in vivo* in liver fibrosis models [19], but it failed in clinical trials due to reduced efficacy and unwanted systemic effects [8].

Others and we have shown that activated HSC abundantly express the platelet derived growth factor receptor (PDGF β R) during liver fibrosis, while its expression is relatively weak on other cells and normal tissues [20–22]. Recently, we have shown that using PDGF β R-specific delivery of IFN γ to activated HSC; acute and advanced liver fibrosis *in vivo* could be significantly inhibited with minimal adverse effects [20,23]. The results of these chimerical constructs of IFN γ and PDGF β R binding moieties were remarkably potent and encouraged us to pursue this strategy and prepare a targeted fusion proteins that are inexpensive and can be feasibly applied in clinical trials.

To that end, we have now produced the recombinant proteins containing a bicyclic PDGF β R-recognizing peptide (BiPPB) fused to IFN γ to synthesize BiPPB-IFN γ or to the signaling moiety of IFN γ (mimetic IFN γ or mimIFN γ) lacking extracellular IFN γ R binding site [24,25] to generate BiPPB-mimIFN γ in *E.coli*. IFN γ and mimetic IFN γ were also expressed in parallel as control proteins. These proteins were analyzed *in vitro* in human HSC cells and *in vivo* in acute liver fibrogenesis mouse model. Encouragingly, we found that the targeted fusion proteins (BiPPB-IFN γ and BiPPB-mimIFN γ) specifically bound to PDGF β R-expressing human HSC and induced significant reduction in major ECM production *in vitro* (collagen). *In vivo*, in the CCl $_4$ mouse model, targeted proteins significantly stimulated the IFN γ -mediated pSTAT1 α -signaling pathway, inhibited collagen accumulation, HSC activation and induced fibrolysis. Apart from therapeutic effects, BiPPB-mimIFN γ and to a lesser extent BiPPB-IFN γ did not affect circulating platelets counts as observed by untargeted IFN γ .

Materials and Methods

Plasmids, Bacterial Strains and Cell Culture

pET42a and pET39b protein expression vectors were purchased from Novagen (Madison, WI, USA). All the restriction enzymes were procured from New England Biolabs (Beverly, MA).

Escherichia coli strain JM109 was used for plasmid propagation and cloning. Strain BL21 (DE3) (Novagen) was used as a host for the production of recombinant proteins. Human hepatic stellate cells, LX2 were kindly provided by Prof. Scott Friedman (Mount Sinai Hospital, New York). LX2 cell line is a well-established human HSC cell line [26]. LX2 cells were cultured in DMEM-Glutamax (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and antibiotics (50 U/ml penicillin and 50 ng/ml streptomycin). Mouse spleen cells, freshly isolated from healthy C57BL/6 mice, were grown in DMEM cell culture medium.

Plasmid Construction

(a) Preparation of pET42a-IFN γ and pET42a-mimIFN γ . Splenocytes (freshly isolated from the spleen of healthy C57BL/6 mice) were seeded in the presence of 20 μ g/ml phytohemagglutinin (PHA) for 24 h. RNA was isolated from spleen cells was used for PCR amplification of mouse IFN γ and mimetic IFN γ using gene specific primers (primer 1, primer 2 for IFN γ and primer 3, primer 4 for mimIFN γ as listed in **Table 1**). The PCR product was purified and digested with Eco RI. The digested gene was inserted into Psh A1/Eco RI digested pET42a vector to produce pET42a-IFN γ or pET42a-mim IFN γ (**Figure S1 and S2**).

(b) Preparation of pET39b-BiPPB-IFN γ and pET39b-BiPPB-mimIFN γ . For synthesis of pET39b-BiPPB-IFN γ and pET39b-BiPPB-mimIFN γ , pET39b-BiPPB was prepared by PCR extension of annealed primers (primer 5, primer 6 and primer 7, primer 8 listed in **Table 1**). The resulting products (2 fragments) were then digested with Bam HI and Not I, purified and ligated to Sca I/Not I digested pET39b vector (**Figure S3**). Thereafter, PCR amplified IFN γ and mimIFN γ (primer 9, primer 10 for IFN γ and primer 11, primer 12 for mimIFN γ listed in **Table 1**) were digested with Not I/Xho I and inserted into Not I/Xho I digested and purified pET39b vector. The resulted recombinant vectors were termed as pET39b-BiPPB-IFN γ and pET39b-BiPPB-mimIFN γ that encodes for BiPPB and IFN γ or mimIFN γ plus a three amino-acid linker (AAA) between them (**Figure S4 and S5**).

All the DNA constructs were confirmed by DNA sequencing. pET42a-IFN γ and pET42a-mimIFN γ expressed proteins of 45.2 and 34.5 kDa respectively including GST tag, His tag, S tag and IFN γ or mimIFN γ . pET39b-BiPPB-IFN γ and pET39b-BiPPB-mimIFN γ

Table 1. Primers used for plasmids construction.

Primers	Sequence	Restriction sites
Primer 1	TGCCACGGCCACAGTCATTGAAAGC	–
Primer 2	GCGAATTCTCAGCAGCGACTCCTTTTC	Eco RI
Primer 3	GCCAAGTTTGAGGTCAACAACCCACAG	–
Primer 4	GCGAATTCTCAGCGACTCCTTTCCG	Eco RI
Primer 5	TGTTCTAGAAACCTCATCGATTGTAAG	–
Primer 6	GCGGATCCCTTACAATCGATGAGGTT	Bam HI
Primer 7	GCGGATCCGGAGGTTGTTACGTAATCTAATAG	Bam HI
Primer 8	TAGCGGCCGCTGAACAATCTATTAGATTA	Not I
Primer 9	GGGCGGCCGCATGTCATGGTACAGTCATTGAA	Not I
Primer 10	GCCTCGAGTTAGCAGCGACTCCTTTTCCG	Xho I
Primer 11	AAGCGGCCGCGAGCCAAGTTTGAGGTCAACAAC	Not I
Primer 12	GCCTCGAGTTATCGACTCCTTTCCGCTCTCTG	Xho I

doi:10.1371/journal.pone.0089878.t001

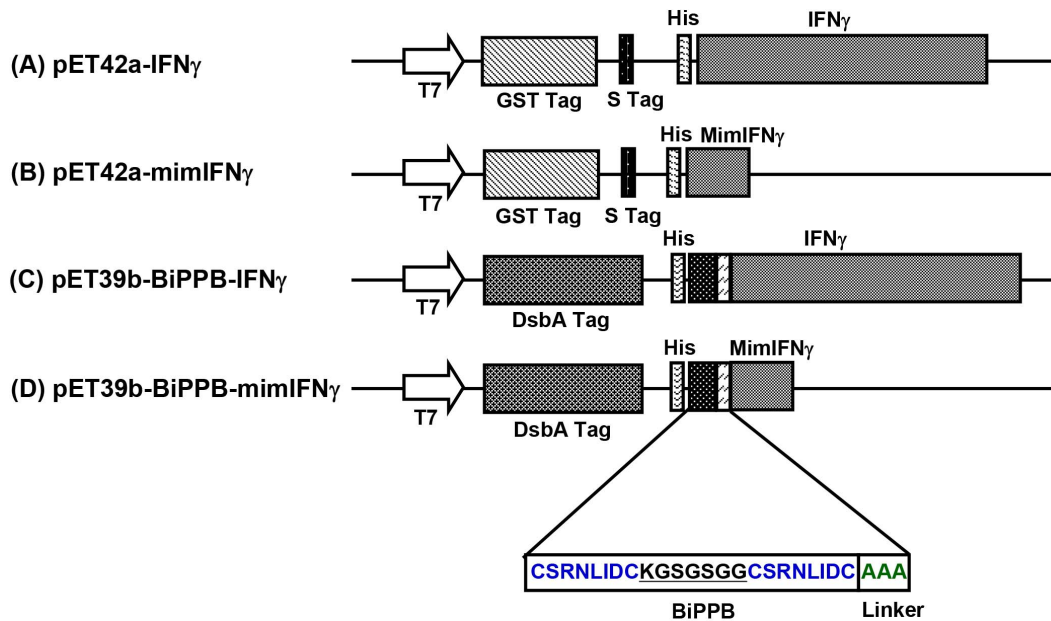


Figure 1. Schematic representation of the prokaryotic vectors used for the expression of the recombinant proteins. IFN γ (A) and mimetic IFN γ (B) were cloned in-frame upstream of His-tag in pET42a (+) vector to achieve cytoplasmic protein expression. The fusion proteins BiPPB-IFN γ (C) and BiPPB-mimIFN γ (D) were expressed in pET39b (+) vector for periplasmic expression of fusion proteins to ensure proper folding and disulfide bonds formation. For the synthesis of fusion proteins, BiPPB was fused to the N-terminal of IFN γ or mimetic IFN γ sequence through a flexible 3 amino acid linker (AAA) maintaining the open reading frame.
doi:10.1371/journal.pone.0089878.g001

expressed 42.8 and 32.2 KDa fusion proteins respectively including Dsb tag, His tag, BiPPB, linker and IFN γ or mimIFN γ .

Expression and Purification of Recombinant Proteins

DNA constructs were transformed into CaCl₂ competent BL21 (DE3) and then induced with 1 mM IPTG at 37°C for 4 h.

For purification of IFN γ and mimIFN γ , the cell pellets were washed and suspended in binding buffer (500 mM NaCl, 20 mM sodium phosphate buffer, 20 mM Imidazole pH7.4), followed by enzymatic lysis (0.2 mg/ml lysozyme, 20 μ g/ml DNase, 10 mM PMSF) for 30 min at 4°C and sonication for 10 min (10–15 short bursts). The cell debris was removed by centrifugation at 12,000 g

for 30 min at 4°C. The supernatant was extensively dialyzed against binding buffer.

For purification of periplasmic proteins BiPPB-IFN γ and BiPPB-mimIFN γ , the cell pellets were suspended in 0.03 mol/l Tris.HCl, 20% sucrose, 0.001 mol/l EDTA, pH8.0. The cells were incubated on ice for 5–10 min followed by centrifugation at 8000 g for 20 min at 4°C. The pellet was then resuspended in ice-cold 5 mM MgSO₄ and stirred for 10 min on ice bath. The cell debris was removed by centrifugation at 8000 g for 20 min at 4°C. The supernatant was extensively dialyzed against binding buffer.

The supernatants were then applied to Ni-charged chelating-Sepharose HisTrap High performance column (Pharmacia Biotech, Uppsala, Sweden). After being washed with binding

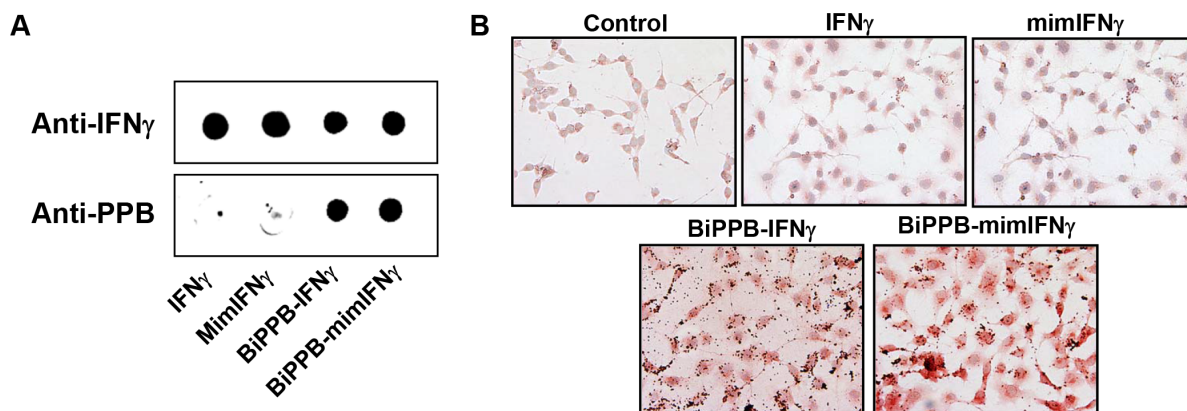


Figure 2. Dot-blot and *in vitro* binding of recombinant proteins in human HSC. (A) Dot-blot analysis of purified recombinant proteins IFN γ , mimetic IFN γ , BiPPB-IFN γ and BiPPB-mimIFN γ using anti-IFN γ and anti-PPB antibody. (B) Representative pictures showing binding of BiPPB-IFN γ and BiPPB-mimIFN γ to human HSC (LX2). Mouse IFN γ and mimIFN γ did not show any binding (similar to control) to human LX2 cells due to species differences and lack of IFN γ R or PDGFR binding sites respectively.
doi:10.1371/journal.pone.0089878.g002

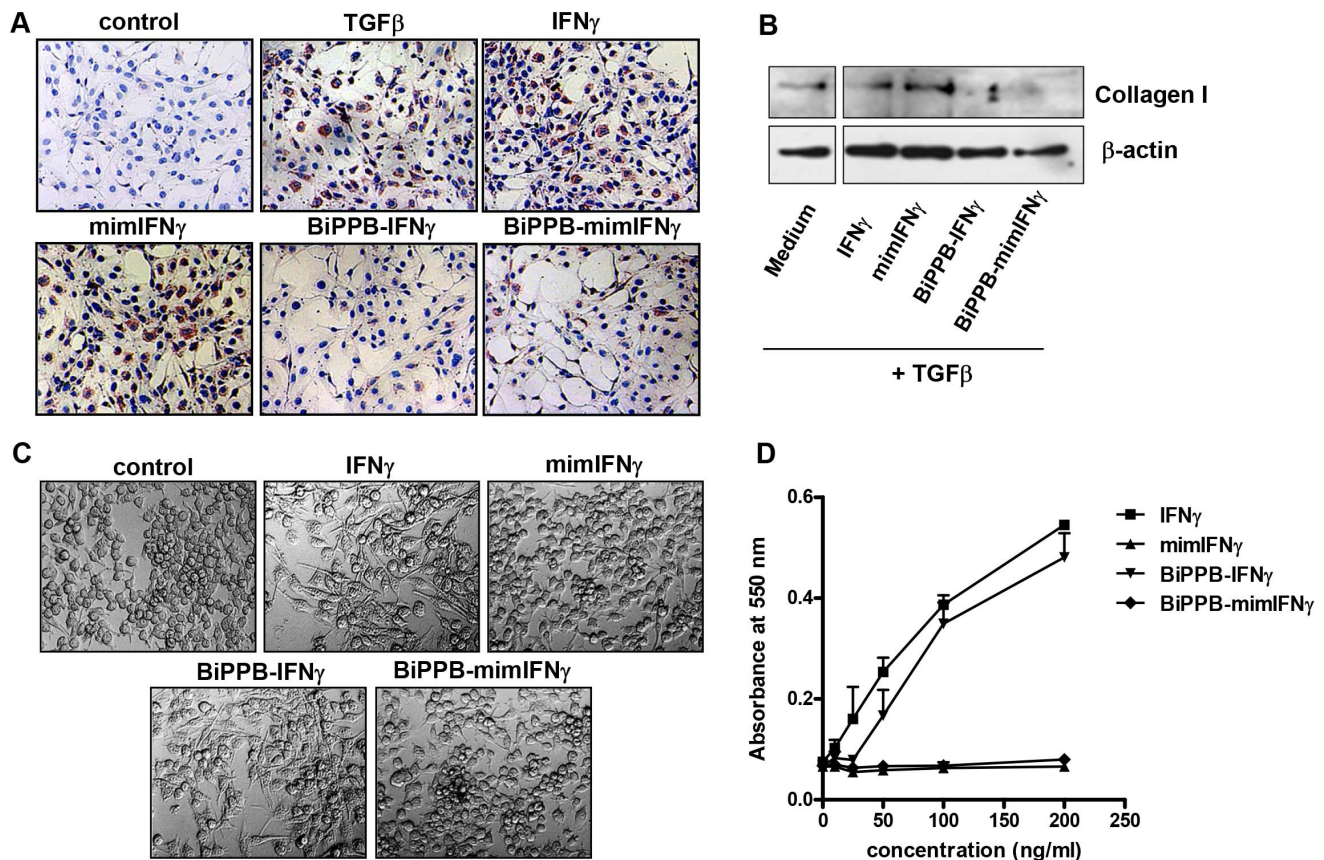


Figure 3. *In vitro* effects of the recombinant proteins in human HSC and mouse macrophages. Representative pictures (A) and western blot analysis (B) of collagen-I stained LX2 cells, incubated with TGF β (5 ng/ml) in combination with different recombinant proteins (1 μ g/ml). In human LX2 cells, only BiPPB-modified proteins attenuated collagen expression, whereas unmodified mouse IFN γ and mimiIFN γ did not cause any reduction due to species restriction and lack of receptor binding sites respectively. (C) Representative microscopic photographs depicting the activation of mouse RAW macrophages after 24 h of incubation with mouse IFN γ and BiPPB-IFN γ (1 μ g/ml) along with 100 ng/ml LPS. (D) Dose-dependent release of nitrogen oxide (NOx) in mouse RAW macrophages after incubation with unmodified IFN γ and BiPPB modified IFN γ fusion protein. MimiIFN γ and BiPPB-mimIFN γ did not induce any NOx release due to absence of IFN γ R binding site and/or lack of PDGFR on RAW macrophages.

doi:10.1371/journal.pone.0089878.g003

buffer, the protein was eluted with the elution buffer containing 500 mM imidazole and then dialysed against PBS buffer overnight.

Dot Blot Immunoassay

Purified proteins were applied on dehydrated PVDF membrane (Roche, Mannheim, Germany) in dot-blot apparatus (Bio-Rad, Hercules, CA, USA). The wells were then incubated with 200 μ l blocking solution (1% BSA in TBS) for 1 h. After washing in TBST (0.05% tween 20 in TBS), the membrane was incubated for 1 h with either anti-IFN γ antibody (1:2000; Abcam, Cambridge, UK) or anti-PPB antibody (1:1000, custom-made, Harlan) followed by 1 h incubation with horseradish peroxidase-labeled secondary antibody. The membrane was washed again with TBST subsequent with incubation in substrate solution (0.06% diaminobenzidine (DAB), 0.08% hydrogen peroxide in TBS) for color development. The membranes were finally rinsed in distilled water and air-dried.

In vitro Binding of Recombinant Proteins in Human HSC (LX2 Cells)

Cells (30,000 cells/well) were seeded in 24-well culture plates (Becton Dickinson, Heidelberg, Germany) and grown overnight at

37°C/5%CO $_2$. Then, cells were incubated with IFN γ , mimiIFN γ , BiPPB-IFN γ or BiPPB-mimIFN γ (1 μ g/ml) for 2 h. Thereafter, cells were fixed and stained using anti-PPB antibody.

In vitro Effects of Recombinant Proteins in Human HSC

Cells were plated in 24 well (30,000 cells/well) and 12 well (75,000 cells/well) culture plates, grown overnight and were starved with 0.5% containing medium for 24 h. Starved cells were then incubated with medium alone, recombinant IFN γ , mimiIFN γ , BiPPB-IFN γ or BiPPB-mimIFN γ (equivalent to 1 μ g/ml) plus 5 ng/ml of human recombinant TGF β 1 (Roche) for 48 h. Subsequently, cells (24 well plates) were fixed and stained for collagen I (1:100, Southern Biotech, Birmingham, AL). In addition, cells (12 well plates) were lysed with 5x SDS-PAGE sample buffer constituted with β -mercaptoethanol (Stratagene, La Jolla, CA) to perform western blot analysis for collagen I (1:250; Southern Biotech) and β -actin (house-keeping gene) as per standard protocols.

Nitric Oxide (NO) Release Bioassay in Mouse RAW Macrophages

The biological activity of recombinant proteins were assessed by measuring the accumulation of nitrite NO $_2$, a stable nitric oxide

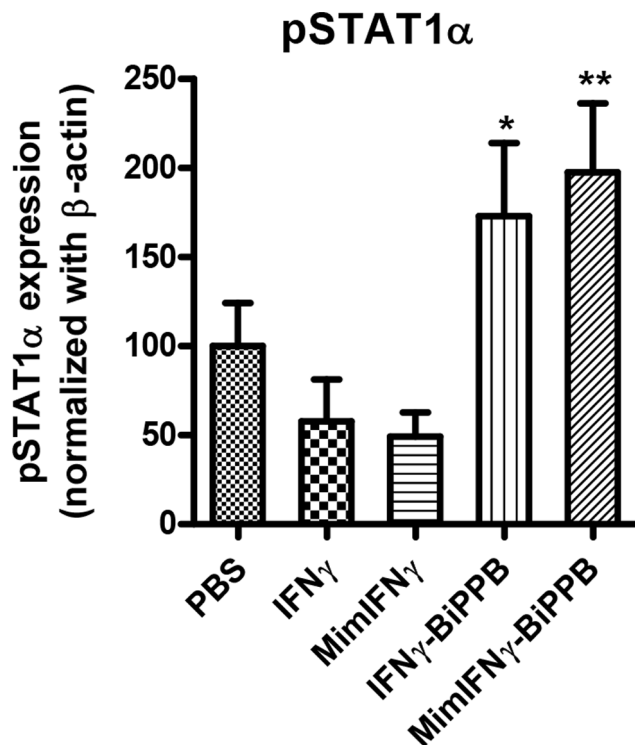


Figure 4. *In vivo* activation of IFN γ pSTAT1 α signaling pathway by recombinant proteins. Western blot analysis of pSTAT1 α after 24 h post intravenous administration of IFN γ (n = 6), MimIFN γ (n = 5), BiPPB-IFN γ (n = 6), BiPPB-mimIFN γ (n = 6) or PBS alone (n = 6). The graph shows significant increase in activation of pSTAT1 α with HSC-targeted fusion proteins (BiPPB-IFN γ , BiPPB-mimIFN γ). Bars represent mean \pm SEM of 5–6 mice per group. *P<0.05 and **P<0.01 versus PBS treated CCl $_4$ mice. The groups were normalized to CCl $_4$ group (treated with PBS).

doi:10.1371/journal.pone.0089878.g004

(NO) metabolite produced by RAW macrophages as described previously [27]. Briefly, cells (1×10^5 cells/200 μ l/well) were seeded in 96-well plates and grown overnight at 37°C/5%CO $_2$. Then cells were incubated with either medium alone or recombinant proteins at different concentrations (10, 25, 50, 100 and 200 ng/ml) together with 100 ng/ml lipopolysaccharides (LPS from E. coli 055:B5, Sigma). After 24 h, the secreted nitrite was measured using Griess reagent (1% sulfanilamide; 0.1% naphthylethylenediamine dihydrochloride; 3% H $_3$ PO $_4$). The absorbance was determined at 550 nm using an ELISA plate reader.

Animal Experiments: Ethics Statement

All the animal experiments were performed in strict accordance with the guidelines and regulations for the Care and Use of Laboratory Animals, University of Groningen, The Netherlands. The protocols were approved by the Institutional Animal Ethics Committee of the University of Groningen, The Netherlands (Permit Number 5429A). Male 6- to 8-week old C57BL/6 mice were purchased from Harlan (Zeist, Netherlands) and kept at 12 h light/12 h dark cycles with ad libitum normal diet.

CCl $_4$ -induced Acute Liver Fibrogenesis Model in Mice

Acute liver injury was induced in male C57BL/6 mice by a single intra-peritoneal injection of carbon tetrachloride (CCl $_4$; 1 ml/kg prepared in olive oil) at day 1. At day 2 and day 3, mice received intravenous injections of IFN γ (n = 6), mimIFN γ (n = 5),

BiPPB-IFN γ (n = 6), BiPPB-mimIFN γ (n = 6) (equivalent to 5 μ g IFN γ /mouse/day) or PBS alone. At day 4, all mice were sacrificed under deep anesthesia by cervical dislocation; blood and livers were collected for subsequent measurements.

In vivo IFN γ (pSTAT1 α) Signaling Pathway

The IFN γ signaling pathway was analyzed in the acute liver fibrogenesis model in mice after 24 h of administration with IFN γ (n = 6), mimIFN γ (n = 5), BiPPB-IFN γ (n = 6), BiPPB-mimIFN γ (n = 6) (equivalent to 5 μ g IFN γ /mouse/day) or PBS alone. Liver tissues samples were homogenized in cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.1% Igepal in 0.5% sodium deoxycholate, 1 tablet of protease inhibitor cocktail and 1 tablet of phosphatase inhibitor in 10 ml) on ice using a tissue homogenizer and the lysates were centrifuged at 12,000 rpm for 1 h at 4°C. 20 μ g of protein was used for western blot analysis performed as per standard protocols using anti-pSTAT1 α antibody (1:1000; cell signaling, Beverly, MA) or anti- β -actin antibody (1:5000; Sigma).

Immunohistochemistry and Immunofluorescence

The cells were fixed with acetone:methanol (1:1), dried and stored until immunostaining. Livers were harvested and transferred to Tissue-Tek OCT embedding medium, and snap frozen in isopentane chilled in a dry ice. Cryosections (4 μ m) were cut using a Leica CM 3050 cryostat (Leica Microsystems, Nussloch, Germany). The sections were allowed to adhere to Superfrost microscopic glass slides (Menzel-Gläser, Braunschweig, Germany), air-dried and fixed with acetone for 10 min. Cells or tissue sections were rehydrated with PBS and incubated with the collagen I antibody (1:100; Southern Biotech), α -SMA antibody (1:600, Sigma) or PPB (1:100; custom-made, Harlan) for 1 h. Thereafter, cells or sections were washed thrice with PBS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 30 min. Cells or sections were washed again and further incubated with HRP-conjugated tertiary antibody for 30 min. Thereafter, peroxidase activity was developed with 3-amino-9-ethyl carbazole (Sigma, St. Louis, MO) for 20 min and nuclei were counterstained with hematoxylin (Fluka Chemie, Buchs, Switzerland). Cells or sections were then mounted with Kaiser's gelatin (Darmstadt, Germany), visualized and photographed using a light microscope (Olympus UK Ltd., Essex, UK).

Quantitative Real-time PCR

Total RNA from liver tissues was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA concentrations were quantitated by a Nanodrop UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (1.6 μ g) was reverse transcribed in a volume of 50 μ l using cDNA synthesis kit (Promega, Madison, WI). All primers were purchased from Sigma-Genosys (Haverhill, UK). Following primers were used, MMP13 forward: CCAGAACTTCCCAAC-CATGT; MMP13 reverse: GTCTTCCCGTGTTCCTCAAA; TIMP1 forward: ATCAGTGCTGCAGCTTCTT; TIMP1 reverse: TGACGGCTCTGGTAGTCCTC; GAPDH forward: ACAGTCCATGCCATCACTGC; GAPDH reverse: GATC-CACGACGGACACATTG. The reactions were performed with 20 ng cDNA using SYBR green PCR master mix (Applied Biosystems, Foster City, CA) according to manufacturer's instructions and were analyzed by ABI7900HT sequence detection system (Applied Biosystems). Finally, the threshold cycles (Ct) were calculated and relative gene expression was normalized with GAPDH (for mouse) as housekeeping gene.

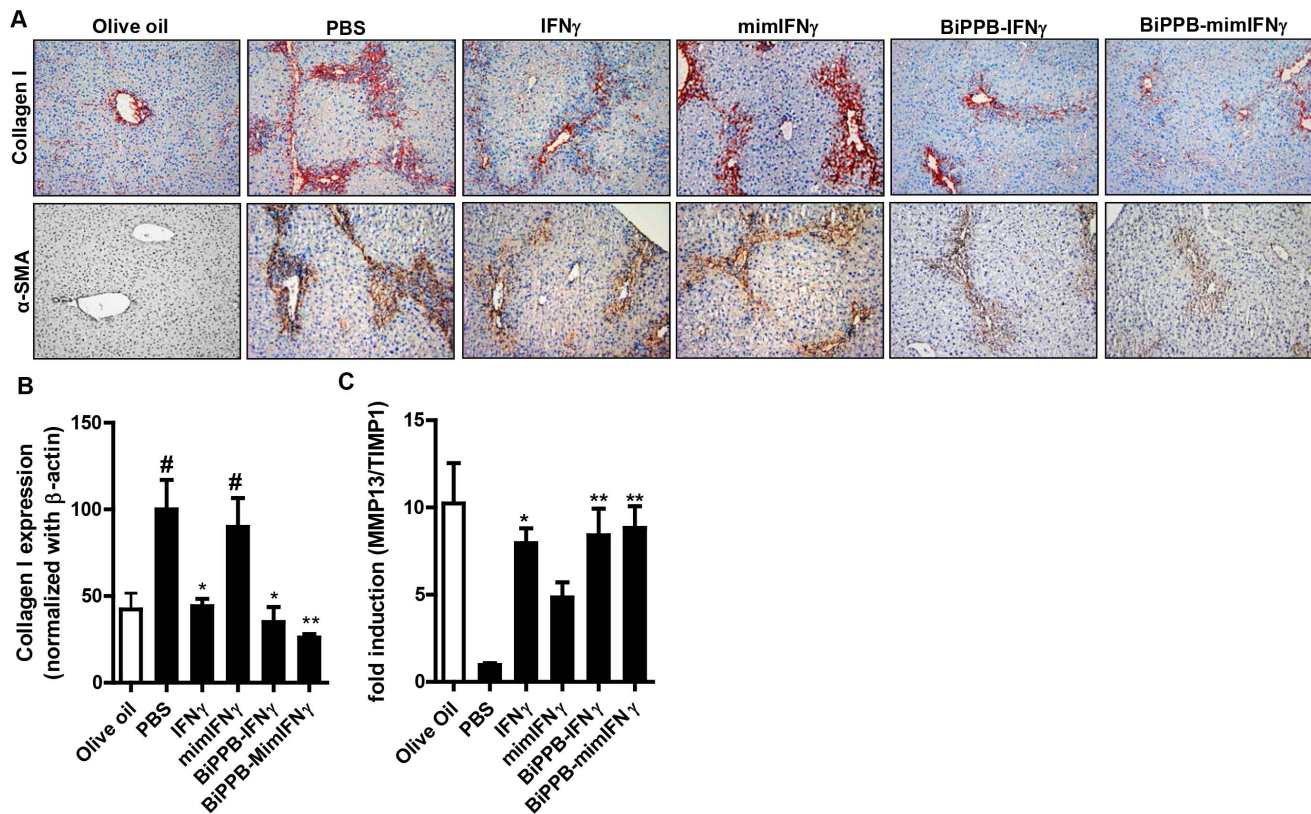


Figure 5. Effects of recombinant proteins on fibrotic parameters in vivo. (A) Representative pictures of collagen I and α -SMA stained liver sections from olive oil treated mice (normal) or CCl $_4$ -treated mice (acute model) that were treated with IFN γ (n=6), MimIFN γ (n=5), BiPPB-IFN γ (n=6), BiPPB-mimIFN γ (n=6) or PBS alone (n=6). Scale bars, 200 μ m. (B) Whole-liver lysates from treated animals were subjected to western blot analysis using anti-collagen I antibody. Graph represents collagen I expression (normalized with β -actin) depicted as mean \pm SEM from n=5–6 mice per group. #P<0.05 denotes significance versus PBS treated olive oil mice and *P<0.05, **P<0.01 denotes significance versus PBS treated-CCl $_4$ mice. For quantitative analysis, the groups were normalized to vehicle group (PBS treated-CCl $_4$ mice). (C) Effect of recombinant proteins on intrahepatic fibrinolysis as determined by the ratio of MMP13 and TIMP-1 transcripts. The groups were normalized to vehicle group (PBS treated-CCl $_4$ mice). Bars represent mean \pm SEM of 5–6 mice per group. *P<0.05, **P<0.01 denotes significance versus PBS treated-CCl $_4$ mice. doi:10.1371/journal.pone.0089878.g005

Statistical Analyses

Data are presented as mean \pm standard error mean (SEM). Multiple comparisons between different groups were performed by one-way ANOVA with Bonferroni post-test using GraphPad Prism version 5.02 (GraphPad Prism Software, Inc., La Jolla, CA, USA).

Results

Construction and Expression of Recombinant Proteins

We synthesized four recombinant proteins: IFN γ , mimetic IFN γ (mimIFN γ), BiPPB-IFN γ and BiPPB-mimetic IFN γ (BiPPB-mimIFN γ) (Figure 1). IFN γ and mimetic IFN γ were expressed using pET42a derived bacterial expression vector to achieve cytoplasmic expression of the proteins (Figure 1A, 1B and Figure S1, S2). While, BiPPB-IFN γ and BiPPB-mimIFN γ fusion proteins were expressed using pET39b (+) expression vector (Figure 1C, 1D and Figure S4, S5) to yield periplasmic expression, required for their proper folding. DNA constructs were extensively analysed for the presence of inserts using specific restriction enzyme digestions and by automated DNA sequencing. Thereafter, constructs were transformed into *E. coli* strain BL21 (DE3) and protein expression was induced by 1 mM IPTG. Proteins of interest with the expected molecular weight were found in total cell pellets. Soluble proteins were purified from the

supernatant of lysed bacterial pellets through Ni-NTA affinity columns. Purified proteins were further dialysed against PBS and concentrated by ultrafiltration. The presence of IFN γ moieties or/and PPB peptides in the prepared fusion proteins was confirmed in dot blots using anti-IFN γ and anti-PPB antibodies (Figure 2A).

In vitro Binding of Recombinant Proteins in Human HSC

The binding of IFN γ to its receptor is strictly species-specific, whereas PDGFR binding is not. In order to demonstrate PDGFR-specific binding of BiPPB containing fusion proteins, we used human LX2 hepatic stellate cells that are known to express PDGFR. The results confirmed the species specificity of IFN γ ; mouse IFN γ and mimetic IFN γ (lacking extracellular IFN γ R binding site) did not show any binding to human HSC (Figure 2B). However, BiPPB-fused mouse IFN γ or mimetic IFN γ proteins showed high binding to human cells (Figure 2B).

In vitro Effects of Recombinant Proteins in Human HSC and Mouse Macrophages

Following the binding studies, we investigated the anti-fibrotic effects of the recombinant proteins in human HSC after their activation with TGF β . In corroboration with the binding studies in human LX2 cells, TGF β -induced collagen expression was strongly inhibited by treatment with the PDGFR-specific BiPPB-IFN γ and

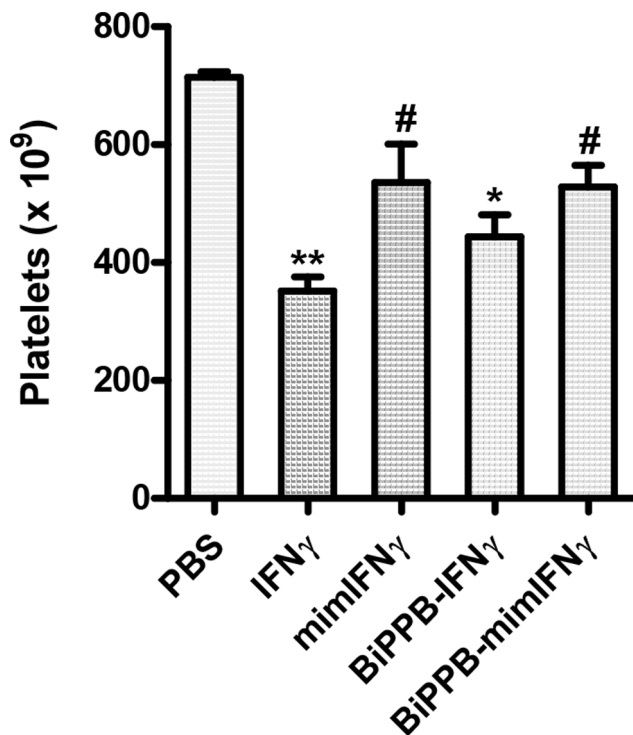


Figure 6. Effects of recombinant proteins on IFN γ -related adverse effect in the acute CCl₄ model. Graph represents platelet counts measured in CCl₄-treated mice receiving PBS (n=6) or the different recombinant proteins IFN γ (n=6), MimIFN γ (n=5), BiPPB-IFN γ (n=6), BiPPB-mimIFN γ (n=6). Bars represent mean \pm SEM of 5–6 mice per group. The results showed a significant reduction in platelet counts following two intravenous administration of IFN γ , which was significantly improved following treatment with BiPPB-mimIFN γ and to a lesser extent with BiPPB-IFN γ . MimIFN γ did not show significant change in platelets counts due to lack of binding to IFN γ R or PDGFR. *P<0.05, **P<0.01 denotes significance versus PBS treated CCl₄ mice. #P<0.05 denotes the significance versus IFN γ -treated CCl₄ mice. doi:10.1371/journal.pone.0089878.g006

-mimIFN γ fusion proteins (Figure 3A, 3B) as analyzed by immunostaining and western blot analysis. While both mouse IFN γ and mimIFN γ did not induce any effect in human cells due to species differences and the absence of a receptor-binding site respectively. On the other hand, mouse IFN γ and BiPPB-IFN γ strongly potentiated LPS-induced activation and dose-dependent release of nitric oxide in mouse RAW macrophages due to presence of mouse IFN γ R (Figure 3C, 3D). However, mimIFN γ lacking the IFN γ R binding site and mimIFN γ -BiPPB directed to PDGFR did not induce any effect due to lack of PDGFR expression on macrophages (Figure 3C, 3D).

In vivo Liver Uptake of BiPPB-modified Fusion Proteins in Acute CCl₄-induced Liver Fibrosis in Mice

Others and we have earlier demonstrated that PDGFR is highly upregulated on activated HSC during acute and advanced liver fibrosis [20,21]. Our group has extensively studied the bio-distribution of proteins modified with PDGFR-recognizing cyclic peptides (PPB), using radiolabeled and imaging studies, and showed high distribution of PPB-modified proteins to PDGFR-expressing hepatic stellate cells in fibrotic livers [23]. In the present study, we assessed the intracellular activation of the pSTAT1 α signaling pathway after 24 h of IFN γ , mimIFN γ , BiPPB-IFN γ and BiPPB-mimIFN γ administration in CCl₄-induced liver fibrosis in

mice. We found a significant increase in pSTAT1 α activation after 24 h of treatment with BiPPB-IFN γ (p<0.05) and BiPPB-mimIFN γ (p<0.01) compared to PBS, IFN γ and mimIFN γ treatments indicating enhanced liver uptake of the fusion proteins due to high PDGFR expression on activated HSC in the fibrotic livers (Figure 4 and Figure S6).

In vivo Effects of BiPPB-modified Fusion Proteins in Acute CCl₄-induced Liver Fibrosis Model in Mice

A single CCl₄ administration induced acute liver injury in mice, characterized by an increased intrahepatic expression of HSC activation marker (α -smooth muscle actin) and an increased deposition of extracellular matrix molecule (collagen I) as shown in Figure 5A–5C. IFN γ , mimIFN γ , BiPPB-IFN γ and BiPPB-mimIFN γ proteins were examined for their anti-fibrotic effects in this experimental model in mice. After two intravenous injections, a strong reduction in collagen I expression was observed with IFN γ (\approx 45% reduction, p<0.05), HSC-targeted BiPPB-IFN γ (\approx 50% reduction, p<0.05) and BiPPB-mimIFN γ (\approx 60% reduction, p<0.01) as analyzed by immunostaining and quantitative western blot analysis (Figure 5A, 5B and Figure S7). Furthermore, α -SMA expression was attenuated by HSC-targeted BiPPB-IFN γ administration and more strongly by BiPPB-mimIFN γ treatment as shown in Figure 5A, while IFN γ had only little effect and mimIFN γ did not induce any reduction in α -SMA expression levels (Figure 5A). Apart from collagen expression and deposition, the balance between collagen degrading matrix metalloproteinases-13 (MMP-13) and their major endogenous inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP-1), is an important determinant of progression or reversal of fibrosis [28,29]. A significant increase in MMP13/TIMP1 transcript ratio was observed after treatment with IFN γ (p<0.05), BiPPB-IFN γ (p<0.01) and BiPPB-mimIFN γ (p<0.01) suggesting activation of fibrolysis and induction of reversal of fibrosis (Figure 5C). Of note, no effect was observed with mimetic IFN γ alone due to lack of an IFN γ R binding site while the inhibitory effects of HSC-targeted BiPPB-modified fusion proteins on collagen deposition were higher compared to non-targeted IFN γ (Figure 5).

The main hurdles in IFN γ -based therapies are the adverse effects that led to the failure of clinical trials. IFN-mediated reduction in blood platelets is clinically relevant and a well-known side effect [30]. Indeed, we observed a significant reduction in the platelet counts following two intravenous treatments with IFN γ (p<0.001), which was significantly improved following treatment with BiPPB-mimIFN γ and to a lesser extent with BiPPB-IFN γ (Figure 6). MimIFN γ did not induce a significant change in platelets counts due to lack of binding to IFN γ R or PDGFR.

Discussion

Hepatocellular damage, hepatic inflammatory cell infiltration and extensive tissue remodeling ultimately culminate into the development of progressive fibrosis and cirrhosis [31]. During liver fibrosis, the interplay between growth factors and cytokines produced by the damaged hepatocytes; inflammatory cells and non-parenchymal cells activate quiescent hepatic stellate cells (HSC). These activated HSC in turn proliferate and accumulate in the injured liver producing large amounts of extracellular matrix proteins [32]. Therefore, therapeutic approaches to silence these activated HSC would be appropriate to inhibit or reverse liver fibrosis [12].

Fibrosis is an heterogeneous process e.g. CCl₄ intoxication results in hepatocyte damage, necrosis, inflammation, and fibrosis,

leading to portal fibrosis or cirrhosis. While bile duct ligation (BDL) stimulates the proliferation of biliary epithelial cells and oval cells, resulting in proliferating bile ductules accompanied by portal inflammation leading to biliary cirrhosis (PBC). As documented in fibrotic animal models (BDL or CCL₄ etc.) and diseased human livers, PDGFR β is expressed abundantly on activated HSC or portal fibroblasts (collectively referred as myofibroblasts-like cells) [21,22]. The expression was found to be very weak in normal healthy tissues or cells (pericytes or smooth muscle cells) relative to expression levels in the fibrotic livers, which motivated us to examine the potential of using PDGFR β for a targeted delivery of anti-fibrotic or apoptotic drugs to the fibrotic livers [12]. In the present study, we have directed IFN γ or mimetic IFN γ (signaling moiety of IFN γ lacking extracellular IFN γ R binding site while retaining activities of IFN γ) [33] to PDGFR β -expressing activated HSC using bicyclic PDGFR β peptides to inhibit liver fibrosis with reduced adverse effects.

IFN γ or mimetic IFN γ modified with bicyclic PDGFR β -recognizing peptide were generated via recombinant technology in *E.coli*. Unmodified IFN γ or mimetic IFN γ were also produced in parallel to serve as respective controls. BiPPB-IFN γ and BiPPB-mimIFN γ were produced from a periplasmic vector (pET39b), since this vector contains the DsbA tag that exports the expressed proteins to the periplasmic space (space between plasma membrane and outer membrane). DsbA, periplasmic enzyme catalyzes the sequential formation of disulfide bonds therefore favors proper folding of the fusion proteins containing bicyclic peptide (cyclized via disulfide bonds) at the N-terminus of IFN γ and mimetic IFN γ . Unmodified IFN γ and mimetic IFN γ were produced using pET42a vector as cytoplasmic proteins. These proteins were expressed, purified and analyzed using immune dot-blot where the presence of IFN γ , mimIFN γ , BiPPB-IFN γ and BiPPB-mimIFN γ was confirmed using anti-IFN γ and anti-PPB antibodies.

The BiPPB-modified proteins were investigated for PDGFR β -specific binding in human HSC. BiPPB-IFN γ and BiPPB-mimIFN γ showed specific binding to PDGFR β -expressing human LX2 cells whereas unmodified mouse derived IFN γ and mimIFN γ did not show binding to these human cells due to species restrictive interaction of mouse IFN γ to mouse IFN γ R, while PDGFR β interaction is not species-specific. BiPPB-IFN γ and BiPPB-mimIFN γ also induced a strong reduction in TGF β -induced collagen I expression in human LX2 cells while IFN γ and mimIFN γ did not affect the collagen expression in these cells corroborating with the binding studies. We did not observe any difference in α -SMA expression *in vitro* after 48 hrs of treatment (data not shown) while significant reduction is observed in collagen expression (**Figure 3A, 3B**), since IFN γ has shown to have direct effect on collagen expression by directly acting on C/EBP β signaling pathway [34] while longer treatments might lead to inhibitory effects on α -SMA expression. These studies clearly demonstrate that directing IFN γ or mimetic IFN γ to the accessory PDGFR β can transform mouse (whose activity is restricted to mouse cells) and mim-IFN γ (unable to enter any cell), into highly active proteins in human LX2 cells. On the other hand, we studied effect of these recombinant proteins in mouse macrophages expressing IFN γ R and lacking PDGFR β . Results showed that IFN γ or BiPPB-modified IFN γ containing IFN γ R binding site activated mouse RAW cells and induced NO release while both mimIFN γ (lacking IFN γ R binding site) or BiPPB-modified mimIFN γ (containing PDGFR β binding site and lacking IFN γ R binding site) did not show any effect in macrophages. These results indicate that targeted mimIFN γ will not influence cell types (especially macrophages which are known to be highly influenced

by IFN γ) other than PDGFR β -expressing cells and therefore will not induce adverse effects in other normal cells or tissues.

In the past years, our group has extensively demonstrated the higher liver accumulation and HSC-specific distribution of PPB-modified proteins [35]. Here we examined the intra-hepatic activation of IFN γ signaling pathway *in vivo* in treated livers to assess the increased accumulation of our targeted proteins in fibrotic livers. IFN γ internalization results in the activation of the JAK-STAT pathway and subsequent phosphorylation of signal transducers and activators of transcription (STAT1) that binds to unique gamma-activated sequence (GAS) regulating IFN γ -responsive genes [36]. Both BiPPB-modified IFN γ and mimIFN γ induced a significant increase in intrahepatic pSTAT1 α signalling compared to unmodified IFN γ and mimIFN γ implicating increased liver accumulation of targeted proteins.

Thereafter, we studied the anti-fibrotic effects of recombinant proteins in the CCL₄-induced liver fibrogenesis model in mice. This model is associated with HSC activation, enhanced PDGFR expression and ECM deposition, the key parameters of fibrogenesis. Two subsequent intravenous injections of IFN γ , BiPPB-IFN γ or BiPPB-mimIFN γ led to the highly significant reduction in collagen I expression (major extracellular matrix protein) and α -SMA (HSC activation marker) expression. Additionally, IFN γ , BiPPB-IFN γ and BiPPB-mimIFN γ enhanced the MMP13/TIMP1 transcripts ratio implying activation of fibrolysis. In this study, we observed anti-fibrotic effects with IFN γ in comparison with our previously reported studies [20,23], is attributed to the increased dose (5 μ g) used here, as compared to 2.5 μ g dose used earlier. MimIFN γ that cannot be internalized due to lack of receptor binding sites did not show any effect on these fibrotic PPB-modified proteins can block the PDGFR *in vitro* [35,37] and this might also account for the observed antifibrotic effects. However, previous studies with PPB-peptides coupled to albumin have shown that this effect does not occur *in vivo* at the doses used [20,23]. To further reinforce this hypothesis, we used synthetic BiPPB as a control but did not observed any effect on the fibrotic parameters (data not shown).

Liver fibrosis or cirrhosis is a slowly progressing disease that develops over many years, therefore patients are treated for longer periods to cure or reverse the disease. Therefore, therapies with improved therapeutic efficacy and preferably without provoking off-target systemic effects would be highly favourable. In clinical trials with IFN γ , patients suffered from mild to severe adverse effects; therefore we investigated one of the well-known IFN-mediated adverse effects, significant reduction in circulating blood platelets (mainly produced by bone marrow) can lead to fatal disorders and also found to be associated with the serotonin levels associated to depression [30]. We found that only two intravenous injections of IFN γ led to a highly significant reduction in platelet counts ($p < 0.001$) in comparison to PBS-treated mice. Furthermore, BiPPB-IFN γ that can still interact with IFN γ R receptor showed a slight reduction in platelet counts ($p < 0.05$), but mimIFN γ (without receptor binding sites) and BiPPB-mimIFN γ (only specific to PDGFR β) did not induce a reduction in platelet counts. Further studies in long-term fibrotic models are important to examine the adverse effects of targeted constructs due to the long-term administration. Since platelets are produced by bone marrow, it is also important to study the effect of these constructs on the bone marrow as these effects may influence other crucial biological processes.

In conclusion, the results in this paper clearly demonstrate that HSC-targeted mimIFN γ is capable of accumulating in PDGFR β -expressing fibrotic livers and exerting an inhibitory effect on fibrotic parameters, which makes this recombinant protein a

highly attractive candidate to explore for the treatment of liver fibrosis. The recombinant synthesis of this chimeric compound will facilitate further translational research with this compound.

Supporting Information

Figure S1 Schematic representation depicting cloning strategy for preparation of pET42a-IFN γ . PCR amplified and EcoRI digested mouse IFN γ gene fragment was cloned in pET42a (+) prokaryotic vector at Psh A1/Eco RI site. (TIF)

Figure S2 Schematic representation depicting cloning strategy for preparation of pET42a-mimIFN γ . PCR amplified and EcoRI digested mouse mimetic IFN γ gene fragment was cloned in pET42a (+) prokaryotic vector at Psh A1/Eco RI site. (TIF)

Figure S3 Schematic representation depicting cloning strategy for preparation of pET39b-BiPPB. BiPPB was prepared by annealing and PCR extension of 2 sets of primers (refer to methods), which were linked together via Bam HI site and cloned in Sca I/Not I digested pET39b (+) prokaryotic vector. (TIF)

Figure S4 Schematic representation depicting cloning strategy for preparation of pET39b-BiPPB-IFN γ . PCR amplified IFN γ prepared for fusion with BiPPB was digested with Not I/Xho I was cloned in pET39b vector at Not I/Xho I site. The resultant recombinant vector termed as pET39b-BiPPB-IFN γ encodes for BiPPB and IFN γ plus a three amino-acid linker (AAA) between them. (TIF)

Figure S5 Schematic representation depicting cloning strategy for preparation of pET39b-BiPPB-mimIFN γ . PCR amplified mimIFN γ prepared for fusion with BiPPB was digested with Not I/Xho I was cloned in pET39b vector at Not I/Xho I site. The resultant recombinant vector termed as pET39b-BiPPB-mimIFN γ encodes for BiPPB and mimIFN γ plus a three amino-acid linker (AAA) between them. (TIF)

Figure S6 *In vivo* activation of IFN γ pSTAT1 α signaling pathway by recombinant proteins. Representative western blot images of pSTAT1 α (upper panel) and β -actin (house-keeping protein, lower panel) after 24 h post intravenous administration of IFN γ (n = 6), MimIFN γ (n = 5), BiPPB-IFN γ (n = 6), BiPPB-mimIFN γ (n = 6) or PBS alone (n = 6). Figure shows the significant increase in activation of pSTAT1 α with HSC-targeted fusion proteins (BiPPB-IFN γ , BiPPB-mimIFN γ). (TIF)

Figure S7 Effects of recombinant proteins on fibrotic parameters *in vivo*. Representative western blot images of collagen I (upper panel) and β -actin (house-keeping protein, lower panel) from CCl₄-treated mice (acute model) that were treated with IFN γ (n = 6), MimIFN γ (n = 5), BiPPB-IFN γ (n = 6), BiPPB-mimIFN γ (n = 6) or PBS alone (n = 6). Figure shows the significant reduction in collagen expression after treatment with HSC-targeted fusion proteins (BiPPB-IFN γ , BiPPB-mimIFN γ). (TIF)

Author Contributions

Conceived and designed the experiments: RB JP KP. Performed the experiments: RB MDR. Analyzed the data: RB JP. Wrote the paper: RB JP KP.

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